Blood-Feeding Behavior of Vesicular Stomatitis Virus Infected *Culicoides sonorensis* (Diptera: Ceratopogonidae)

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ABSTRACT To determine whether vesicular stomatitis virus (VSV) infection of *Culicoides sonorensis* Wirth & Jones (Diptera: Ceratopogonidae) affects subsequent blood-feeding behavior, midges injected with either virus-infected or virus-free cell lysates were allowed to blood feed for short (10-min) or long (60-min) periods on 2, 3, and 4 d postinoculation (DPI). Generalized linear mixed models were fit to test the effects of infection status, duration of feeding period, and DPI on the percentage of females that blood fed. VSV-infection significantly reduced the percentage of females that blood fed on 2 DPI, the day of peak virus titer. On 3 DPI a significantly greater percentage of midges blood fed when allowed 60 min to feed. This effect was not seen on 2 and 4 DPI and was not dependent on VSV infection status. The impact of changes in blood-feeding behavior by infected insects on virus transmission is discussed.

KEY WORDS *Culicoides sonorensis*, vesicular stomatitis virus, behavior, generalized linear mixed model

Vesicular stomatitis virus (family *Rhabdoviridae*, genus *Vesiculovirus*, VSV) causes sporadic epizootics in cattle, horses, and swine in the western United States. Clinical signs of vesicular stomatitis are indistinguishable from those of foot and mouth disease, and it is therefore an Office of International Epizootics Listed, reportable disease. Although it has been shown that several insect species can transmit or become infected with VSV (Ferris et al. 1955; Shelokov and Peralta 1967; Comer et al. 1990; Mead et al. 1997, 1999; Nunamaker et al. 2000, 2003; Drolet et al. 2005; Perez de Leon et al. 2006; Perez de Leon and Tabachnick 2006), once animals are infected, the virus can be spread through direct contact (Hanson 1981). Insects are likely to be important in initiating VSV epizootics by acting as bridge vectors between unknown reservoir systems and domestic livestock or as maintenance reservoirs themselves through vertical transmission from infected females to offspring (Tesh et al. 1972; Comer et al. 1990, 1994). They also may be important in the spread of virus among livestock herds (Price and Hardy 1954).

Culicoides sonorensis Wirth & Jones (Diptera: Ceratopogonidae), formerly *Culicoides variipennis sonorensis* (Holbrook et al. 2000), feeds primarily on livestock (Raich et al. 1997), and it has been implicated in the transmission of several animal pathogens in the western United States, including bluetongue virus (Price and Hardy 1954, Tabachnick 1996) and epizootic hemorrhagic disease virus (Foster et al. 1977). *C. sonorensis* has been shown to be susceptible to infection with VSV in laboratory studies (Nunamaker et al. 2000, Drolet et al. 2005), and it is capable of transmitting virus to mammalian hosts, including guinea pigs and cattle (Perez de Leon et al. 2006, Perez de Leon and Tabachnick 2006). Field evidence of potential *C. sonorensis* involvement with VSV transmission includes several virus isolations from field-collected insects (Walton et al. 1987, Francy et al. 1988, Kramer et al. 1990, Mead et al. 1999).

The dogma that arthropod-borne pathogens have little or no adverse effect on the arthropods that transmit them has been disproved for numerous virus–vector systems. Some detrimental effects on the arthropod vector include cytopathic effects on specific organ systems (Mims et al. 1966, Weaver et al. 1988, Bowers et al. 2003, Girard et al. 2007), reduced blood-feeding success (Mims et al. 1966, Grimstad et al. 1980, Platt et al. 1997), decreased fecundity (Turell et al. 1985), decreased survival (Lee et al. 2000, Moncayo et al. 2000), and delayed development of immature life stages (Beaty et al. 1980, Turell et al. 1985, Lee et al. 2000). Additionally, the infection of specific tissues, such as the nervous system or salivary glands may have very direct consequences on the behavior of the insect and its ability to find a host and take a bloodmeal.

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The idea that infection can alter vector behavior to increase transmission has been examined for several vector-borne pathogens (Molyneux and Jefferies 1986, Moore 1993, Hurd 2003, Schaub 2006). Subtle differences in host seeking, feeding, and reproduction can have enormous impact on the success of pathogen transmission. One common result observed for virus-infected hematophagous insects is increased probing time and number of probes per blood feed (Grimstad et al. 1980, Platt et al. 1997). This could lead to greater transmission potential for the virus if increased probing corresponds to more hosts probed before a meal is taken, greater probability of pathogen delivery, or leads to a larger pathogen exposure dose. Alternatively, it could result in decreased transmission if increased host contact leads to greater exposure to defensive behaviors that prevent feeding or increase mortality.

The current study determines whether VSV infection adversely affects the ability of *C. sonorensis* to blood feed. Blood-feeding success for midges intrathoracically inoculated with either virus-infected or virus-free cell lysates were compared for two feeding times and three different postinjection time periods.

Materials and Methods

Virus and Cells. Domestic VSV-New Jersey strain 82-34333, a bovine isolate from a 1982 VSV outbreak in Idaho, was grown in Vero MARU (VM; Middle America Research Unit, Panama) cells and medium 199 with Earle's salts (M199-E, Sigma, St. Louis, MO). At 24 h postinfection, the master stock was aliquoted, frozen, and stored at −80°C. From this master stock, one aliquot was passaged an additional time in VM cells and aliquoted to create a working stock with a titer of 3.0×10^8 plaque-forming units (PFU) per milliliter. One aliquot of this working stock was used per experiment as infectious inocula. Aliquots of uninfected VM cells in M199-E media were made, frozen, and stored at −80°C, and these were used as negative control virus-free cell lysate inocula.

Insects. *C. sonorensis* pupae were harvested from an established colony of midges from Idaho (AK) (Jones and Foster 1974, 1978). For each experimental group, ≈1,000 pupae were placed on moistened cotton in a 1-pint (0.67-liter) paper emergence carton and held at 25–27°C until emergence (1–2 d). Adult female midges were inoculated intrathoracically at 0–1 d postemergence with either virus-infected or virus-free cell lysate by using microinjection needles double pulled from 5-μl glass capillary tubes and mounted on 27-gauge needles. Injections were deemed complete once the abdomen visually distended. Insects were held in paper cartons at 25°C with a photoperiod of 11:13 (L:D) h. Midges were provided a 10% sucrose solution ad libitum, which was removed 18–24 h before blood-feeding attempts.

Virus Assays. Virus was titered from subsamples (n = 11–15) of injected insects on days 0–10 to determine virus replication rates. Insects were frozen at −80°C on respective days postinjection (DPI). Frozen insects were ground in 200 μl of gnat antibiotic medium and titered by standard plaque assay on VM cells as described previously (Drolet et al. 2005).

Insect Feeding. At 2, 3, or 4 DPI, midges were anesthetized with CO_2 for 10 s and transferred to feeding cones in groups of 28–144 midges per cone. Time points of 2, 3, and 4 DPI were chosen to include optimal midge feeding (typically 2–3 d postemergence) and peak virus titer. Insects were offered a bloodmeal consisting of defibrinated sheep blood in an artificial feeder using a reinforced silicone membrane (Davis et al. 1983, Hunt and McKinnon 1990). After allowing midges to feed for either 10 or 60 min, insects were removed from the blood source, anesthetized for 10–15 s with CO_2, and sorted as to blood-feeding status on a chill table.

Each combination of treatments (i.e., virus-infected or virus-free cell lysate-injected midges blood fed on 2, 3, or 4 DPI for either 10 or 60 min) was repeated three to five times to account for variation in midge feeding behavior. Fifty-two trials were conducted over 22 separate days, and on each day at least one virus-infected and one virus-free cell lysate-injected trial was completed. The original experimental design planned to examine holding times of 2 and 4 DPI, but after initial results showed distinct differences in feeding behavior between 2 and 4 DPI, it was decided to also examine a holding time of 3 DPI. When examining 2 and 4 DPI holding times, only two trials were conducted per day; one VSV-injected and one virus-free cell lysate-injected group for a given feeding duration. When examining the 3 DPI holding time, all trials were conducted on the final 4 d of the experiment, with four trials conducted per day; one VSV-injected and one virus-free cell lysate-injected group for each of the feeding durations. Therefore, the manner in which data were collected for 3 DPI differed from data collected for 2 and 4 DPI. To avoid false conclusions, data collected for 2 and 4 DPI were analyzed separately from data collected for 3 DPI.

Statistical Analysis. Statistical analyses were made for measurements of insect blood-feeding behavior related to infection status, duration of feeding time, and DPI. The number of midges that fed on the bloodmeal in each experiment was modeled as a binomial count, and these were fitted to a generalized linear mixed model by using SAS PROC GLIMMIX (Littell et al. 2006). Two models were fitted for analysis, one for data collected at 2 and 4 DPI and the other for 3 DPI. These were fitted separately to reflect differences in the manner in which data were collected as described above under Insect Feeding. Replicate effects were combined as random effects.

Results

Feeding success was determined for *C. sonorensis* inoculated with VSV-infected or with virus-free cell lysates (infection status) after exposure to an artificial membrane feeder for either 10 or 60 min (duration) at 2, 3, or 4 d DPI. The total sample size for each treatment group ranged 246–579 midges. The majority

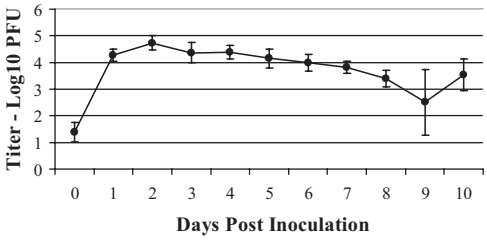

Fig. 1. Virus titer graphed as the average $\log_{10}$ PFU per midge. For each time point, 11–15 midges were assayed. Virus titer peaked at 2 DPI and then gradually decreased over time. Error bars represent the SD of virus titers for individual insects.

of midges (>99%) were either fully fed or did not feed at all, so partially fed individuals were not included in the analysis.

Virus Titers. The average ± SD quantity of virus injected per insect was 1.4 ± 0.37 $\log_{10}$/midge based on virus titers measured for 11 insects that had been injected and immediately frozen and stored at −80°C (day zero time point). To determine the standard growth curve of virus over time in intrathoracically inoculated *C. sonorensis*, the average titer was calculated for 11–15 insects at each time DPI. The average peak virus titer was 4.7 ± 0.28 $\log_{10}$/midge, ≈2,000 times greater than that of the initial inoculum, and occurred at 2 DPI (Fig. 1). There was a steady decline in virus titer after the peak at 2 DPI.

Blood-Feeding Behavior. Statistical analysis using a generalized linear mixed model revealed that feeding success varied with VSV infection status and approached significance when holding times of 2 and 4 DPI were combined ($F = 4.14$; df = 1, 14; $P = 0.06$). Significant differences in blood-feeding success also were observed for the interaction of 2 and 4 DPI and VSV infection status ($F = 7.20$; df = 1, 14; $P < 0.05$). For a holding time of 3 DPI, feeding duration had a significant effect on blood-feeding success ($F = 16.42$; df = 1, 9; $P < 0.005$).

Infection Status. The effect on blood-feeding success seen for the interaction of hold time and infection status was further analyzed statistically by comparing percentages of fed females between VSV-injected and virus-free cell lysate-injected insects for each hold time. Figure 2A shows that the effect is largely due to the greater percentage of females that blood fed from the virus-free cell lysate-injected group (70.0%) compared with the VSV-injected midges (48%) on 2 DPI ($F = 13.04$; df = 1, 14; $P < 0.005$). At 4 DPI, the percentage of fed females for both groups was not significantly different (60 and 63%, respectively).

Blood-Feeding Duration. When blood feeding was examined across hold time and infection status, significantly more females blood fed when allowed 60 min than 10 min to feed at a hold time of 3 DPI (82 ± 3 and 42 ± 10%, respectively) (Fig. 3), but not at hold times of 2 or 4 DPI. The observed difference in blood-feeding percentages based on feeding duration was not dependent on VSV infection status (Fig. 2B).

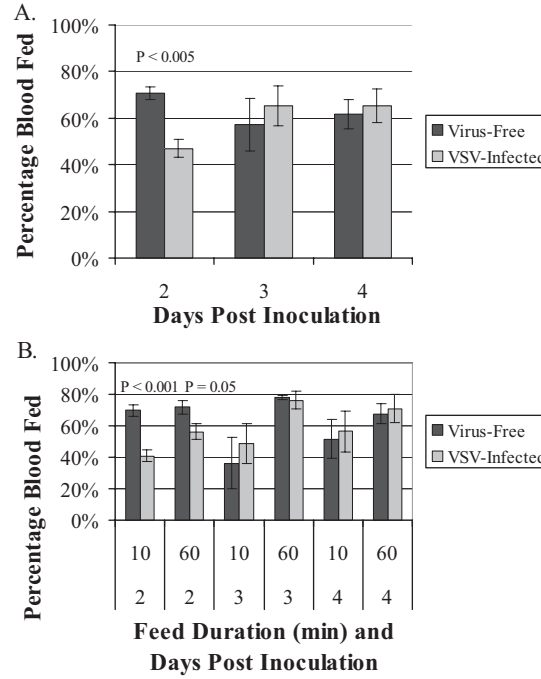

Fig. 2. Percentages of VSV-injected or virus-free cell lysate injected insects blood fed on 2, 3, or 4 d DPI for all data by DPI (A), or subdivided by feeding duration (10 or 60 min) and DPI (B). Error bars represent the SEM. *P* values are provided where results were significant.

Infection Status and Blood-Feeding Duration. Although the model did not detect a significant difference in feeding percentages based on feeding duration for midges fed 2 DPI, this time point was examined further to determine whether feeding duration had an effect on the feeding success of infected midges. In both 10- and 60-min feeds, a significantly larger percentage of uninfected midges fed than infected midges; however, the magnitude of this difference was less for midges allowed 60 min to feed. When given 10 min to feed, a 28% difference in the percentage of females

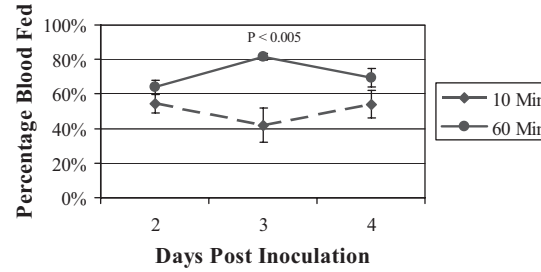

Fig. 3. Feeding behavior based on feeding duration (10 or 60 min) displayed DPI across infection status. Because no significant difference in feeding behavior was observed between VSV-injected and virus-free cell lysate-injected midges based on feeding duration, data from infected and uninfected midges were combined. *P* values are provided where results were significant.

that blood fed was seen between uninfected (69%) and infected (41%) midges. This was significant by a Student's unpaired *t*-test with a two-tailed distribution ($t = 5.47$, df = 8, $P < 0.001$). When allowed 60 min to feed, only a 16% difference was seen between uninfected (71%) and infected (56%) midges that blood fed ($t = 2.27$; df = 8; $P = 0.05$). Despite the difference in feeding success observed for 10- and 60-min feeds at 2 DPI between infected and uninfected midges, results indicate that infected midges did not take significantly longer to feed than uninfected midges at the time scale tested.

Discussion

Observed and model-predicted percentages of fully fed midges agreed well among trials with both models used for statistical analysis. For both model fits combined, the mean absolute difference between observed and predicted results was 0.012 with a standard error of 0.002. The maximum absolute difference between observed and predicted results was 0.056. There was no indication of a poor fit as determined from examination of residuals. Conclusions drawn from the statistical analyses accomplished by separating 2 and 4 DPI data from 3 DPI data were not different from those drawn when all data were combined, but by using better fit models, the statistics more accurately reflected the manner in which the data were collected.

C. sonorensis exhibited altered blood-feeding avidity in response to infection with VSV. A transient decrease in the percentage of females blood feeding was observed at 2 DPI between infected and uninfected midges. Insects infected with virus can sometimes take longer to successfully blood feed (Grimstad et al. 1980, Platt et al. 1997, Schaub 2006). For *Aedes aegypti* (L.), the increased time it took for dengue virus-infected females to blood feed was primarily due to an extended period of probing on the host, and to some extent to longer host location and actual engorgement (Platt et al. 1997). In the current study, the paucity of insects found partially fed suggested that it is not likely that the actual act of blood feeding takes longer, but rather that factors leading up to obtaining a bloodmeal are affecting blood-feeding success. Additionally, because no significant difference between infected and uninfected midges was found based on feeding duration, VSV infection of *C. sonorensis* did not cause a detectible delay in feeding at the time scale used (10 versus 60 min).

A significant difference in the percentage of females that blood fed based on feeding duration was detected on 3 DPI when infected and uninfected midge data were combined. Midges allowed 60 min to feed fed much better than those given only 10 min (Fig. 3). It is unclear why such a difference was observed only on 3 DPI. Midges allowed 60 min on 2 and 4 DPI fed slightly better than those allowed 10 min, but these differences were not significant. Although feeding duration significantly affected feeding success at 3 DPI, no significant difference was found between infected and uninfected midges fed for 10 or 60 min. Therefore, it seems that infection with VSV does not affect the speed at which *C. sonorensis* feeds.

Any alteration in blood-feeding behavior, including blood-feeding success, could have a significant impact on midge survival and virus transmission, even if it only occurs transiently. Survival could be affected if the insect incurs greater risk through increased contact time with a potential host or is unable to successfully obtain a bloodmeal. Virus transmission could be enhanced whether a delay in feeding is due to increased probing before becoming engorged, or whether it involves an increase in the movement of infected midges from host to host before actually blood feeding. Transmission could be decreased if host-seeking behavior is inhibited.

The significantly decreased feeding behavior observed for infected midges corresponded to the peak virus titer in midges at 2 DPI (Fig. 1). After peaking on day 2, virus titers decreased gradually, but they were not significantly different among the 3 days examined (days 2, 3, and 4). By 9 DPI, virus had cleared or significantly decreased in titer in several insects tested. Growth kinetics of virus has been examined in orally infected midges over a longer time course, and variability in the virus titer continues as long as midges are maintained (unpublished data). It is possible that this variability is a result of individual differences in the ability of midges to control or clear the virus.

It is important to acknowledge that intrathoracic inoculation of insects likely results in an altered virus growth curve as compared with infection per os, and therefore may not represent chronologically how a natural infection would affect blood-feeding behavior. Determining the time course of tissue infection after intrathoracic inoculation would be helpful in making the connection between tissue tropisms and chronological infection patterns with the results in this study. This could then be compared with previous work on VSV tissue tropism and infection time course in *C. sonorensis* after oral infection (Drolet et al. 2005). That the oral infection rate of *C. sonorensis* for VSV-NJ in artificial membrane feedings, as measured by virus isolation assays, is highly variable (36% at 7 d postfeed in one study [Drolet et al. 2005] and 24% at 3 d postfeed and 6.7% at 7 d postfeed in another [Nunamaker et al. 2000]), makes it difficult to test behavior of group fed midges after per os infection. Additionally, because *C. sonorensis* prefer collective feeding of dense populations in feeding cages, it is difficult to look at behavior of individual midges.

For insects fed VSV-NJ-spiked bloodmeals through artificial membrane feeders, one previous study found that insects fed a bloodmeal with a titer of 9 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ were found to have a range in titer of 5.3–5.8 $\log_{10}$ $TCID_{50}$ immediately after feeding, 4.1–5.1 $\log_{10}$ $TCID_{50}$ at 1 d postfeed, and 3.8–5.3 $\log_{10}$ $TCID_{50}$ at 3 d postfeed (Nunamaker et al. 2000). Another study found that after feeding on a titer of 1×10^9 PFU/ml VSV-NJ, seven of 10 midges were infected with a range in titer of 1.7–3.5 $\log_{10}$ PFU per insect and by day 7 postfeed, 15 of 42 insects were infected, with a virus titer of 0.3–5.5 $\log_{10}$ PFU per

insect (Drolet et al. 2005). The range of titers found for individual insects immediately after inoculation with a stock VSV-NJ suspension of 3.0×10^8 plaque PFU/ml for the current study was 0.8–1.7 $\log_{10}$ PFU (average 1.4 ± 0.37) per insect. The peak virus titer for injected midges occurred at 2 DPI and ranged 4.2–5.2 $\log_{10}$ PFU per insect (average 4.7 ± 0.28). Although this was higher than reported previously values for orally infected insects at 2 d postinfectious feed, it was within the range of infection rates that have been observed after viral dissemination.

Previous studies have found that viruses can damage the insect tissues they infect (Mims et al. 1966, Weaver et al. 1988, Bowers et al. 2003, Girard et al. 2007). Severe damage to tissues pertinent for blood feeding, such as salivary glands or neural tissues responsible for regulating blood feeding, could cause decreased blood-feeding success; however, because the altered blood-feeding behavior was transient and observed at 2 DPI but not at subsequent holding times, tissue damage is unlikely to be the cause. Moreover, a previous study found that per os infection of *C. sonorensis* with VSV caused no detectible damage to insect tissues despite virus dissemination from the midgut (Drolet et al. 2005). Regardless, infection through intrathoracic inoculation could possibly result in an altered pathogenesis and will therefore be examined in the future.

Because virus–vector systems are dynamic, and given that changes in blood-feeding behavior do occur in VSV-infected midges, it is important to understand how this might be important when other factors that affect transmission are also considered. Transovarial transmission (TOT) of VSV has been shown to occur in sand flies and has been implicated as a possible overwintering mechanism for the virus (Tesh et al. 1972, Comer et al. 1990, Comer et al. 1994). If TOT of VSV occurs in *C. sonorensis*, midges would be infected as newly emerged adults, and virus could affect behavior during a first blood feed. This study looked at 2 DPI primarily because nulliparous midges tend to feed best at this time point postemergence, but if TOT occurs, it might be important for virus transmission as well. Additionally, if virus infection leads to a delay in blood feeding and subsequent egg maturation, infection may progress to developing eggs enhancing the potential for vertical transmission in the first gonotrophic cycle as well as in subsequent cycles. This could have a significant impact on virus maintenance.

This study has demonstrated that the infection status of VSV injected *C. sonorensis* midges was a statistically significant factor in affecting the percentage of females that blood fed. The specific factors that cause decreased blood-feeding percentages in infected females could have very different effects on virus transmission. The use of a slower growing virus, or a lower initial virus titer for injections, may help resolve whether observed differences in percentages of blood-fed females on 2 DPI are due to virus titers or to physiological factors in the insect. If the decline in blood-feeding success is due to a problem with host seeking or decreased insect activity, transmission would be adversely affected. Alternatively, if it is due to an increase in probing time, number of probes per feed, or number of hosts probed before feeding, it could be advantageous for virus transmission as long as the increase in exposure to host bite avoidance behavior or immune defense systems did not result in increased insect mortality. Understanding the effects of changes in blood-feeding behavior of infected insects is important in developing a complete picture of virus transmission and epidemiology.

Acknowledgments

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